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2) **The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury.**
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Department of Biochemistry and Molecular Biology, University College London, London, UK.
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4) **The mitochondrial permeability transition pore and its role in cell death.**
Crompton M
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5) **The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins.**
Marzo I; Brenner C; Zamzami N; Susin S A; Beutner G; Brdiczka D; Remy R; Xie Z H; Reed J C; Kroemer G
Centre National de la Recherche Scientifique, Unite Propre de Recherche 420, F-94801 Villejuif, France.
Journal of experimental medicine (UNITED STATES) Apr 20 **1998** , 187 (8) p1261-71, ISSN 0022-1007 Journal Code: 2985109R

6) Cyclophilin - D **binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore.**
Crompton M; Virji S; Ward J M
Department of Biochemistry and Molecular Biology, University College London, UK. m.crompton@bsm.biochemistry.ucl.ac.uk
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Involvement of cyclophilin D in the activation of a mitochondrial pore by Ca^{2+} and oxidant stress

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Heart and liver mitochondria contain a structure that is able to form a large non-selective pore in the inner membrane under conditions of high matrix Ca^{2+} and oxidant stress. The pore is blocked by cyclosporin A (CSA). In this study, rat liver mitochondria were covalently labelled with a photoactive CSA derivative in the presence and absence of the pore ligands Ca^{2+} and ADP. Photolabelling of a 21-kDa protein was selectively depressed by Ca^{2+} in a manner reversed by ADP. The protein exhibited peptidylprolyl *cis-trans* isomerase (PPIase) activity and was inhibited by CSA (K_i 8 nM). The PPIase was associated with the outside of sonicated submitochondrial particles but dissociated in 0.5 M NaCl. When mitochondria were treated with increasing concentrations of digitonin, the 21-kDa PPIase fractionated with the matrix marker enzyme, malate dehydrogenase. A second PPIase of 18 kDa fractionated with the intermembrane-space marker, adenylate kinase. Photolabelling of the 18-kDa PPIase was unaffected by Ca^{2+} or ADP. The 21-kDa PPIase was digested with endoproteinase Asp-N and 11 of the peptides were N-terminally sequenced. The sequences were most similar to those of human cyclophilin-D, and it is concluded that this protein is probably the CSA receptor during pore blockade by CSA. The implications of these findings are discussed.

Keywords: calcium; oxidant stress; cyclophilin.

Cyclophilins (CyP) form a family of cyclosporin A (CSA)-binding proteins which catalyse the interconversion of *cis* and *trans* isomers of peptidylprolyl bonds in peptides and proteins [1]. The peptidylprolyl *cis-trans* isomerase (PPIase) activity is blocked by CSA. Cyclophilins accelerate the folding of proteins for which isomerisation of peptidylprolyl bonds is rate limiting *in vitro* [2] and, possibly, *in vivo* [3, 4]. Cyclophilins may operate with chaperones. For example, CyP-40 associates with the 90-kDa heat-shock protein (Hsp90) [5], and a 156-kDa surface protein which has a CyP-like domain contains PPIase and chaperone activities [6]. Not all aspects of cyclophilin action relate to PPIase activity since the CyP – CSA complex inhibits the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, whereas cyclophilins themselves are inactive in this respect [7, 8]. FK506-binding proteins (FKBP) form another class of PPIase [1]. FKBP-12 associates with the Ca^{2+}-release channel of sarcoplasmic reticulum and influences the channel-gating properties [9]. Thus PPIases may participate widely in the facilitation of protein conformational change.

A cyclophilin may also participate in a structure that forms a large non-selective pore in the mitochondrial inner membrane. The pore is normally closed but opens when activated synergistically by high intramitochondrial Ca^{2+} and oxidative stress [10, 11]. It is blocked by ATP, ADP and CSA [10, 11]. An open pore of 2-nm diameter is indicated [12, 13], and attention has focussed largely on the possible role of the pore in cell injury [11, 14]. Recent *in vivo* measurements have detected pore opening during myocardial reperfusion injury [15], which is associated with tissue-Ca^{2+} overload and oxidative stress, and during oxidant-stress-(hydroperoxide)-induced injury in hepatocytes [16]. Furthermore CSA provides protection against reoxygenation and hydroperoxide-induced injury in heart and liver cells [17–20]. Several observations indicate that blockade of the pore by CSA reflects the involvement of cyclophilin in pore opening/closure [21–23]. We utilised a tritiated photoactive CSA derivative to covalently label the relevant protein. Photolabelling of a 22-kDa PPIase in heart mitochondria was selectively modified by other pore ligands [24], thereby suggesting that this particular protein is involved. In the present study, a similar protocol was applied to liver mitochondria, and the results indicate an involvement of the same protein. Partial amino acid sequencing reveals a close similarity of the protein to human CyP-D, and localisation studies indicate that it associates with the matrix face of the inner membrane. The significance of these findings are discussed.

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Abbreviations. CSA, cyclosporin A; PA-CS, photoactive cyclosporin; CyP, cyclophilin; PPIase, peptidylprolyl *cis-trans* isomerase; FKBP, FK506-binding protein; SMP, submitochondrial particles.

Enzyme. Peptidylprolyl isomerase (EC 5.2.1.8).

MATERIALS AND METHODS

Labelling and analysis of mitochondrial membranes with a photoactive CSA derivative. Mitochondria were isolated from male Sprague-Dawley rat livers [24] and suspended in medium A (210 mM mannitol, 70 mM sucrose, 10 mM Tris/HCl, pH 7.2). The mitochondria were photolabelled within 1 h of

preparation with a tritiated photoactive derivative of CSA (Sandoz 212-122; designated PA-CS). The photolabelling protocol used differed from that used previously with liver mitochondria [25] by incorporation of modifications used subsequently with heart mitochondria [24]. Since the covalently bound label is susceptible to esterase-catalysed deconjugation, the modified procedure involved the inclusion of the esterase inhibitor, phenylmethylsulfonyl fluoride, at all stages and the omission of silicone-oil washes. The inclusion of other esterase inhibitors did not improve the recovery of photolabelled products from heart [24] or liver mitochondria. The silicone-oil washes, which were used to remove unconjugated photolabel, were omitted since deconjugation occurred even in the presence of phenylmethylsulfonyl fluoride.

Mitochondria (20 mg protein/ml) were incubated at 0°C for 30 min in the presence of 50 μM $CaCl_2$ or 1 mM EGTA to allow Ca^{2+} repletion/depletion of the matrix space. PA-CS (1 μM) and ADP (1 mM, when indicated) were added. After 5 min in the dark, incubates were irradiated with long ultraviolet light (Mineralight lamp, model UVGL-58) for 10 min. The mitochondria were washed once in medium A and suspended in 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.8, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride. These protease inhibitors were included in all subsequent fractionation steps. The photolabelled mitochondria were sonicated and membranes were isolated as described [24]. Photolabelled membranes (20 mg protein/ml) were extracted with 100 mM Chaps in 150 mM NaCl, 10 mM Hepes, pH 7.4, 1 mM EGTA and protease inhibitors for 30 min on ice. Extracts were clarified (120000 g, 40 min) and fractionated by gel filtration in the same medium containing 10 mM Chaps. SDS/PAGE was performed and proteins were extracted as described previously [24]. Molecular-mass standards were ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; chymotrypsinogen, 24 kDa; lysozyme, 14 kDa; cytochrome *c*, 13 kDa; and aprotinin, 6 kDa.

Purification of PPIases (CyP-21 and CyP-18). PPIase was assayed by means of the peptide *N*-succinylalanylalanylprolylphenylalanyl-4-nitroanilide [21]. PPIases were purified from soluble (supernatant) and membrane fractions. The same purification procedures were used except that membranes were first extracted in medium containing 100 mM Chaps (as described above) and 10 mM Chaps was included in all purification media. In general, samples prepared from 400–900 mg mitochondrial protein were diluted in 10 mM Hepes, 1 mM EGTA, protease inhibitors, pH 7.8 (medium B) to give [NaCl] or [KCl] of less than 30 mM, and applied to an S-Sepharose column (31 ml). All PPIase activity was retained. PPIase was eluted in a single step with medium B plus 300 mM NaCl. The eluate was diluted 10-fold and fractionated on a Mono-S column (8 ml, Pharmacia) in medium B by means of an NaCl gradient. Eluted PPIase was concentrated by single-step elution (300 mM NaCl) from a small (1-ml) Mono-S column then fractionated twice by gel filtration on a Superdex-75 column (Pharmacia) equilibrated in 150 mM NaCl, 1 mM EGTA, 10 mM Hepes, protease inhibitors, pH 7.4. To purify Cyp for the purposes of identification, greater than 95% of PPIase eluted at each step was passed on to the next step; for sequencing purposes, rather less was retained at each step.

Protein sequencing. Proteins were separated by SDS/PAGE, stained with Coomassie Blue and digested in excised gel pieces by endoproteinase Asp-N (Boehringer). Peptides were recovered by means of sonication and applied directly to Aquapore AX-300 (30 mm×2.1 mm) and OD-300 (150 mm×2.1 mm) columns connected in series on a Hewlett-Packard 1090M HPLC system. The columns were developed with a linear acetonitrile gradient in 0.1% trifluoracetic acid, and peptide elution was monitored by means of diode-array detection (200–600 nm). Fractions were collected and applied to an Applied Biosystems 477A pulsed-liquid automated sequencer, modified as described [26].

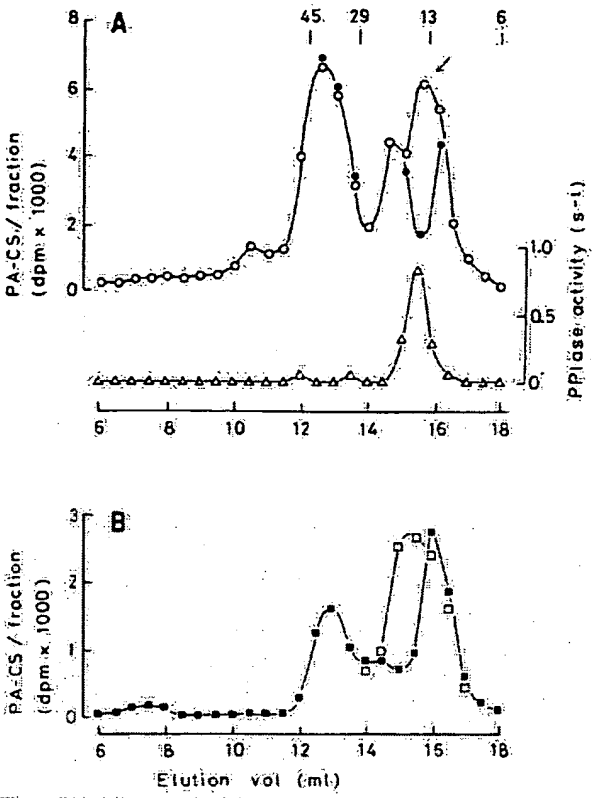

Fig. 1. The influence of Ca^{2+} and ADP on photolabelling of mitochondrial membrane components. (A), mitochondria were covalently labelled with PA-CS in the presence of EGTA (○) or Ca^{2+} (●). Membrane extracts were analysed by gel filtration (Superose-12, Pharmacia; calibrated with 6–45-kDa markers). The PA-CS-labelled contents of all fractions were determined. △, PPIase activity in extracts of membranes prepared from mitochondria containing 52 mg protein. (B), mitochondria were photolabelled in the presence of Ca^{2+} (■) or Ca^{2+} and 1 mM ADP (□). Membrane extracts were fractionated as in (A).

Other methods. Submitochondrial particles (SMP) were prepared by sonication of mitochondria in 10 mM Tris/HCl, 0.1 mM EDTA, protease inhibitors, pH 7.8, for 2 min. SMP were recovered by sedimentation (120000 g, 30 min). Mitochondria were fractionated by means of addition of digitonin in medium A [27]. The suspension was stirred gently on ice for 15 min and then centrifuged (8000 g, 10 min). From the recovery of marker enzymes (Figs 4 and 5) the supernatant was taken as the intermembrane-space fraction and the pellet (mitoplasts) as the inner-membrane/matrix fraction. Malate dehydrogenase, adenylate kinase, monoamine oxidase and succinate dehydrogenase were assayed by means of standard protocols [27].

RESULTS

The effect of pore ligands on photolabelling of Cyp-21 by PA-CS. CSA-binding proteins were covalently labelled by means of a tritiated, photoactive derivative of CSA (PA-CS) [28]. Fig. 1 A shows a typical photolabelling profile after separation of membrane constituents by gel filtration. In spite of its

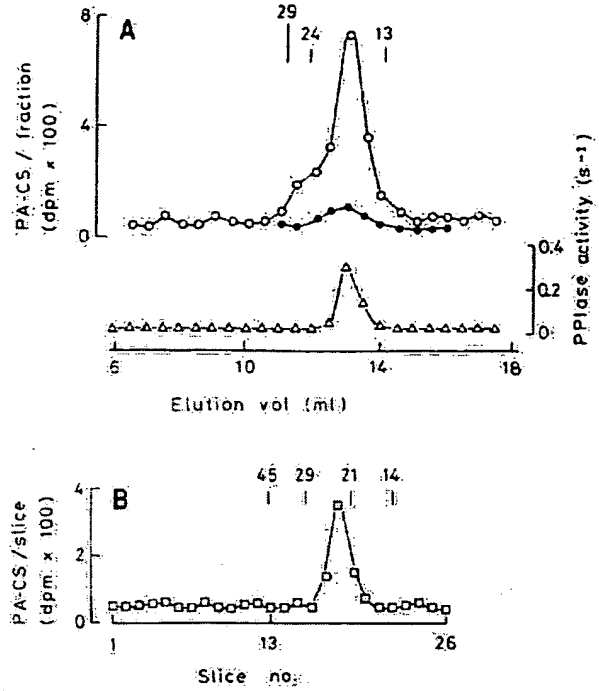

Fig. 2. Gel filtration of the component that was photolabelled in a Ca^{2+}-sensitive manner. (A), mitochondria were photolabelled and fractionated as in Fig. 1 in the presence of either EGTA (○) or Ca^{2+} (●). The Ca^{2+}-sensitive fractions (indicated by an arrow in Fig. 1A) were analysed by gel filtration on a Superdex-75 (Pharmacia) column. The elution positions of molecular-mass markers (13–29 kDa) are shown. (△), PPIase activity. (B), fractions from (A), from mitochondria fractionated in the presence of EGTA, that contained PPIase activity (elution vol., 13–13.5 ml) were analysed by SDS/PAGE. The amounts of radioactivity present in 2-mm gel slices are shown.

intrinsic selectivity for CSA-binding proteins [24] PA-CS labelled a spectrum of components, and further selection criteria were therefore needed. We made use of the finding that Ca^{2+} not only activates the pore but also inhibits interaction of CSA with the relevant pore component. Photolabelling in the presence of Ca^{2+} selectively reduced the amount of radiolabel that eluted at 15.5 ml (Fig. 1A); elsewhere the radiolabelling profiles were essentially the same. In four such experiments with different mitochondrial preparations, Ca^{2+} selectively decreased photolabelling of the 15.5-ml fraction by 66 ± 13% (mean ± SEM). Pore activation by Ca^{2+} is antagonised by ADP, and this effect offers a further potential criterion for selection. The low incorporation of radiolabel into the 15.5-ml fraction in the presence of Ca^{2+} was reversed when ADP was included during the photolabelling. In three similar experiments with separate mitochondrial preparations, ADP selectively increased photolabelling of this fraction in the presence of Ca^{2+} by 215 ± 38% (mean ± SEM). The enhancement of photolabelling by ADP appears to be largely a reversal of the Ca^{2+} effect since in the presence of EGTA ADP increased photolabelling of this fraction to a lesser extent (44 ± 11%, mean ± SEM; three experiments). The mechanism of action of Ca^{2+} and ADP are unresolved but the finding that these pore ligands have opposite effects on pore activation and on radiolabelling of this particular fraction suggests that this fraction contains the relevant CSA-binding protein.

Refractionation of the Ca^{2+}-sensitive fractions on Superdex-75 gave a peak of radiolabel at approximately 18 kDa with samples from Ca^{2+}-free mitochondria and negligible radiolabel with samples from Ca^{2+}-replete mitochondria (Fig. 2A). Other experiments (data not shown) confirmed that fractions from mitochondria photolabelled in the presence of ADP and Ca^{2+} yielded a peak of radiolabel at 18 kDa similar to that from Ca^{2+}-free samples. In general there was also a small higher-molecular-mass shoulder to the main 18-kDa peak. As with heart mitochondria [24] continuous deconjugation of bound photolabel occurred in spite of the inclusion of a range of protease inhibitors and esterase inhibitors, and this deconjugation prevented further purification of the protein in radiolabelled form. Nevertheless, sufficient radiolabel was retained to allow analysis of the 18-kDa peak by SDS/PAGE, and this analysis yielded a radiolabelled band at approximately 22 kDa (Fig. 2B). This value is similar to that of CyP-21 if we consider that the radiolabel would contribute approximately 1.5 kDa. In addition, the Ca^{2+}-sensitive fractions corresponded to the peak in PPIase activity on both types of gel-filtration column (Figs 1A and 2A). The PPIase was inhibited greater than 95% by CSA with a K_i value of 8 nM (data not shown). The data are consistent with the Ca^{2+}/ADP-sensitive protein in liver mitochondria being CyP-21.

We also analysed the H_2O-soluble fraction after sedimentation of SMP. As described previously [25], this fraction contained two photolabelled components of approximately 18 kDa and 90 kDa (on Superdex-75 gel filtration). The 18-kDa compo-

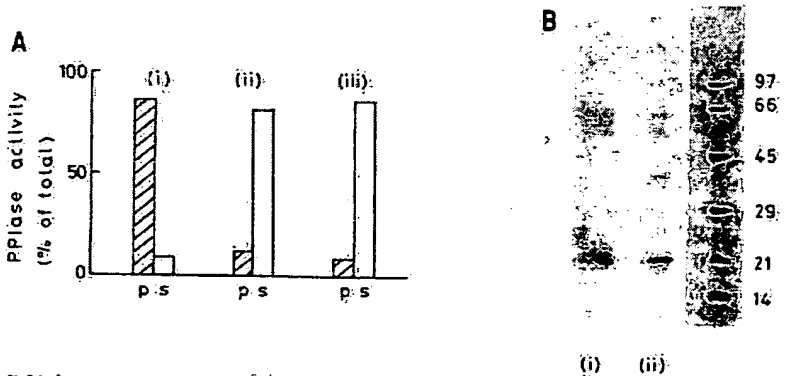

Fig. 3. Dissociation of CyP-21 from membranes in high salt. (A), SMP were suspended in 20 mM Hepes, pH 7.4, 0.5 mM EGTA, protease inhibitors with the following additions: (i) none; (ii) 500 mM NaCl; (iii) 500 mM NaCl and sonication. SMP were centrifuged and the PPIase activities of the supernatants (s) and pellets (p) determined. Activities are expressed as a percentage of that measured in SMP suspended in medium containing 30 mM Chaps. (B), the PPIase of the supernatant and pellet of (A iii) were purified and analysed by SDS/PAGE. The positions of molecular-mass markers (14–97 kDa) are shown. (i), PPIase of salt-washed membranes; (ii), PPIase of salt-wash supernatant.

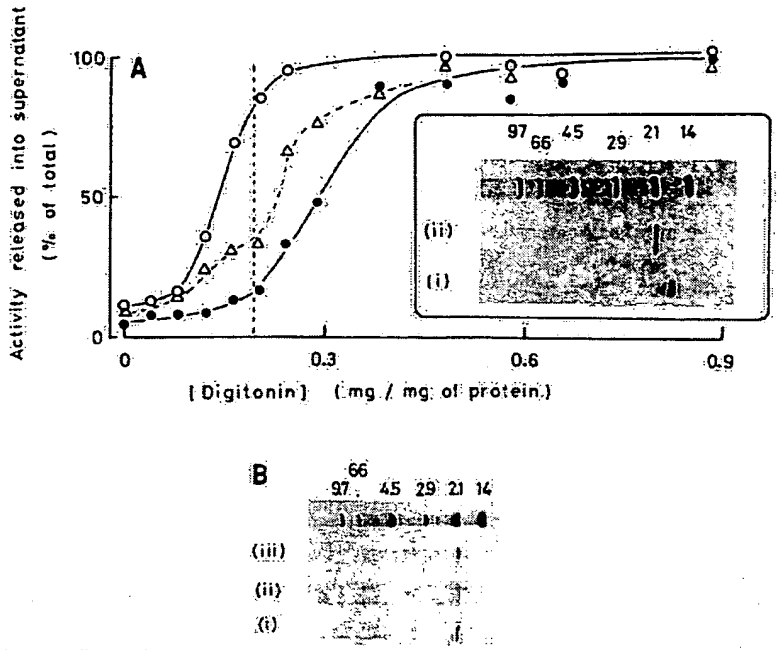

Fig. 4. Separation of CyP-21 from CyP-18 by means of digitonin fractionation. (A), mitochondria in medium A were treated with digitonin and centrifuged. The activities of adenylate kinase (○), malate dehydrogenase (●) and PPIase (△) in the supernatant were expressed as a percentage of their activities in whole mitochondria treated with 30 mM Chaps. Mitochondria treated with 0.19 mg digitonin/mg mitochondrial protein (dashed line) were centrifuged. The PPIase of the supernatant and the pellet (mitoplasts) were purified and analysed by SDS/PAGE (inset). The positions of molecular-mass markers (14–97 kDa) are shown. (i), supernatant PPIase; (ii), mitoplast PPIase. One representative experiment of four is shown. (B), mitoplasts were isolated as in (A), suspended in 500 mM KCl, 10 mM Hepes, pH 8.0, 0.5 mM EGTA, protease inhibitors and centrifuged. The supernatant (cytoplasmic-side salt wash) was retained. The salt-washed mitoplasts were suspended in the same medium, sonicated and separated into membranes and supernatant (matrix-side salt wash). The PPIase in each of the three fractions was purified and analysed by SDS/PAGE. (i), salt-washed membranes; (ii), cytoplasmic-side salt wash; (iii), matrix-side salt wash.

nent eluted with PPIase activity, purification of which yielded CyP-18. In two experiments photolabelling of neither the 18-kDa component nor the 90-kDa component was markedly affected by ADP or Ca^{++} (i.e., less than 20%).

The intramitochondrial location of CyP-21. The location of CyP-21 in SMP was investigated as reported in Fig. 3. Most of the PPIase activity remained membrane bound in low-salt medium (Fig. 3A), but the activity largely dissociated from the membrane in the presence of 500 mM NaCl, irrespective of whether the SMP were resonicated or not (Fig. 3A). Purification of the total PPIase from soluble and membrane fractions after salt treatment produced a 21-kDa band on SDS/PAGE (Fig. 3B). It appears that CyP-21 binds electrostatically to the outside of SMP; since the inner membrane becomes inverted during preparation of SMP the outside corresponds to the matrix side of the inner membrane.

Although not detected in SMP from liver (Fig. 3B) or heart [24], mitochondrial preparations contain CyP-18 [29, 30]. The intramitochondrial location of CyP-21, and its relation to that of CyP-18, was investigated by means of digitonin fractionation. Relatively low [digitonin] selectively lyse the mitochondrial outer membrane and leave the inner membrane largely intact, but at higher [digitonin] both membranes are lysed. Adenylate kinase and malate dehydrogenase were used as markers to quantify protein release from the intermembrane space and matrix respectively (Fig. 4A). Release of total PPIase as the [digitonin] was varied differed from the release of either marker (Fig. 4A). The releases of CyP-21 and of CyP-18 were determined at 0.19 mg digitonin/mg mitochondrial protein (Fig. 4A), a concentration that gave the best separation between intermembrane-

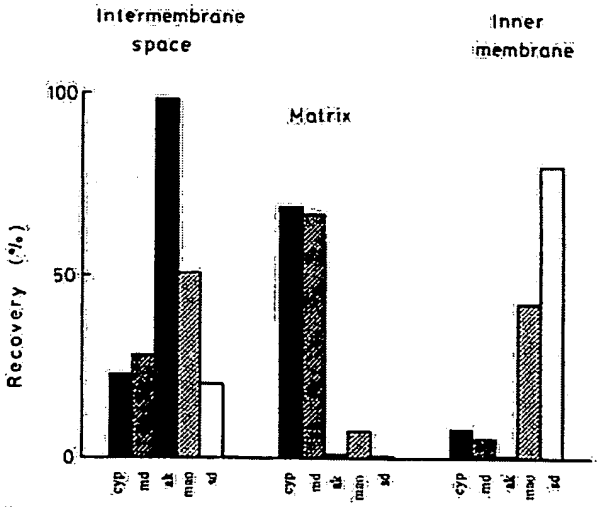

Fig. 5. The intramitochondrial location of CyP-21. Mitochondria were treated with 0.19 mg digitonin/mg protein and centrifuged. The supernatant (s) was retained. The mitoplast pellet was fractionated as in Fig. 4B into cytoplasmic-side salt wash (c), matrix-side salt wash (m) and salt-washed membranes (p). The fractions were assayed for CyP-21 (cyp), malate dehydrogenase (md), adenylate kinase (ak), monoamine oxidase (mao) and succinate dehydrogenase (sd). Intermembrane space, (s) plus (c); matrix, (m); inner membrane, (p).

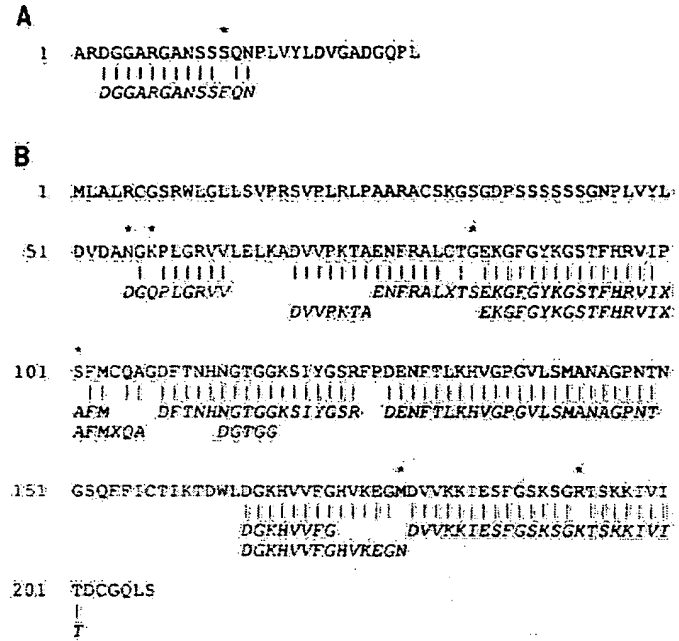

Fig. 6. CyP-21 peptide sequencing. Amino acid sequences were obtained after digestion of purified CyP-21 with endoproteinase Asp-N. The enzyme cleaved at some glutamic acid in addition to aspartic acid residues. Amino acid residues that differ from those of previously identified sequences are marked with an asterisk, unidentified residues are designated X. (A), the amino acid sequence of one peptide (shown in italics) was found to be nearly identical to the previously defined N-terminal sequence of a rat liver mitochondrial CyP [30]. (B) The amino acid sequences of the remaining ten sequences (shown in italics) are aligned with the full-length sequence of human CyP-D [31].

space enzymes and inner-membrane/matrix (mitoplast) enzymes. Purification of the total PPIase from each fraction (Fig. 4A) revealed that the mitoplasts contained only CyP-21, and the intermembrane-space fraction contained only Cyp-18.

To analyse the distribution of CyP-18 and CyP-21 more precisely, the mitoplasts were treated with 500 mM KCl to remove any material bound to the external face of the mitoplast membrane (cytoplasmic-side salt wash). This treatment released some PPIase, identified as CyP-21 (Fig. 4B), although the amount was minor. This material and the post-mitoplast supernatant were taken as the intermembrane-space fraction. Only CyP-21 was detected in the cytoplasmic-side salt wash and only CyP-18 in the post-mitoplast supernatant. The separation of the two activities in this way was observed consistently (four experiments) and allowed the amounts of CyP-18 and CyP-21 in the intermembrane space to be estimated from the initial PPIase activities of the post-mitoplast supernatant and cytoplasmic-side salt wash, respectively. The reason for this separation is not clear, but since both enzymes are H_2O soluble it seems that CyP-21 associates more strongly with the membrane than CyP-18 under these particular conditions. CyP-21 is the more basic of the two proteins, as indicated by its greater retention on cation-exchange columns (CyP-18 eluted at approximately 60 mM NaCl and CyP-21 at 95 mM NaCl from a 8-ml Mono-S column). Thus, the two isoforms were also easily separable by cation-exchange chromatography. Similarly, only CyP-21 was detected in the matrix fraction and in the salt-washed membranes (Fig. 4B), and the initial PPIase activities could be used to estimate the CyP-21 contents of these fractions.

The distribution of CyP-21 after digitonin fractionation was compared (Fig. 5) with that of marker enzymes, monoamine oxidase (outer membrane), succinate dehydrogenase (inner membrane), malate dehydrogenase (matrix) and adenylate kinase (intermembrane space). An outer-membrane-containing fraction is not specified since the marker, monoamine oxidase, partitioned almost equally between the intermembrane-space and inner-membrane fractions. The recoveries of CyP-21 in the three fractions were almost identical to those of malate dehydrogenase, and distinct from those of the other markers. This distribution was representative of three such experiments in which the recoveries of CyP-21 in the intermembrane space and matrix fractions (19±2% and 74±4%, respectively; means±SEM) resembled those of malate dehydrogenase (17±6% and 78±7%, respectively) rather than adenylate kinase (76±10% and 21±9%, respectively). It is concluded therefore that CyP-21 is a matrix enzyme (Fig. 5) which associates electrostatically with the matrix face of the inner membrane (Fig. 3).

In contrast, in four experiments (Fig. 4A), CyP-18 was detected only in the intermembrane-space fraction, and may be tentatively assigned to this fraction. This finding was not pursued since photolabelling of CyP-18 was unaffected by Ca^{2+} or ADP. We estimate that CyP-18 accounted for 22±6% of total PPIase activity (mean±SEM, four experiments), the remainder being due to CyP-21.

Identification of CyP-21 as CyP-D. Fig. 6 reports the partial amino acid sequence of CyP-21. With the exception of one peptide, a search of data bases revealed 96% sequence identity of peptides to human CyP-3 [31], also designated CyP-D [1]. The best alignments of the same CyP-21 peptides with other CyP isoforms yielded the following sequence identities: 79% to rat CyP-A [32], 66% to human CyP-B [31], and 60% to rat CyP-C [33]. CyP-A is a cytosolic isoform [1], whereas CyP-B and CyP-C have been located in endoplasmic reticulum [1, 34]. Human CyP-D was thought to be a possible mitochondrially located enzyme because of its N-terminal targetting sequence (predicted from its cDNA) [31]. Subsequently, Connern and Halestrap sequenced, by means of Edman degradation, the N-terminus of a CyP from rat liver mitochondria [30]. Except for

one residue, this sequence is identical to that of the peptide of CyP-21 that could not be aligned with human CyP-D (Fig. 6A) which suggests that this sequence is located in the N-terminal region. We were unable to sequence the undigested protein since it was N-terminally blocked, which might indicate an additional unresolved sequence at the N-terminus of the mature protein.

From the sequence information it seems that the human and rat CyP-D proteins diverge considerably in the N-terminal region. There is little discernable sequence similarity among mitochondrial targetting sequences and the divergent N-terminus might represent the remnant of such a sequence. A smaller CyP was isolated from rat liver mitochondria (17–18 kDa; which presumably corresponds to CyP-18 of the present study) in which the divergent N-terminal region (ten amino acids) was absent, which suggests that the protein may have arisen by cleavage of the larger isoform [30]. However, CyP-21 would not appear to be an intermediate since most of the mitochondrial CyP is present in this form and a residual targetting sequence seems improbable in a mature protein. Full sequence information of both isoforms is needed before any conclusions can be drawn about whether the larger isoform is processed to the shorter form. This is especially so if, as indicated here, the two isoforms are located differently when (pre)sequence differences would be expected.

DISCUSSION

Several observations suggest the involvement of a cyclophilin in the Ca^{2+}-activated inner-membrane pore. Early studies reported that there was a correlation between the number of mitochondrial CSA-binding sites associated with the pore and with PPIase activity [21, 35] and that cyclosporins A, G and H inhibit the pore and PPIase with similar relative potencies [22]. These measurements were made without knowledge of there being more than one mitochondrial CyP and refer to composite activities. Cyclophilins were not implicated by earlier photolabelling experiments with liver mitochondria [25]. But evidence was subsequently obtained for involvement of a 22-kDa PPIase in heart mitochondria by means of a modified photolabelling protocol [24]. This protocol resolved the anomaly between liver and heart mitochondria, and the PPIase has been identified as CyP-D (Fig. 6).

The photolabelling approach is based on evidence that CSA interaction with the pore-related component is markedly affected by the other pore ligands Ca^{2+} and ADP. There are indications that Ca^{2+} depresses CSA interaction with the pore component [13, 36]. Thus, our finding that Ca^{2+} depresses PA-CS photolabelling of liver mitochondrial CyP-D is consistent with this protein being the target for CSA when CSA blocks the pore. Ca^{2+} activation of the pore in mitochondria [10] and patch-clamped membranes [37] is antagonised by ADP. ADP also acts synergistically with CSA to cause pore closure in metabolite-depleted mitochondria [25, 38]. The observed suppression by ADP of the effects of Ca^{2+} on CyP-D photolabelling is also in accordance therefore with an involvement of this protein in pore function.

We have been unable to detect any effect of Ca^{2+} (500 μM) or ADP (1 mM) on the K_i value for CSA inhibition of the PPIase activity of purified CyP-D from liver or heart (K_i = 5–9 nM; this study, [24]). Thus the isolated enzyme is Ca^{2+}-insensitive and ADP-insensitive. Sensitivity to these agents must be conferred by another protein, which presumably binds Ca^{2+} and, possibly, ADP. Since Ca^{2+} and ADP are recognised pore ligands by virtue of their abilities to activate and inhibit the pore, respectively, the data provide evidence that CyP-D associates with a component of the pore. CyP-D is a matrix protein (Figs 4 and 5) and it is specifically matrix Ca^{2+} that depresses pore-component interaction with CSA and causes pore activation [36]. Thus, the photolabelling data support the following events in the matrix compartment, in accordance with previous suggestions [21, 35]:

$$Ca^{2+} + \text{target-protein} \cdot \text{Cyp} \rightleftharpoons \text{Cyp} \rightleftharpoons \text{CyP} \cdot \text{CSA}$$

where CyP-D binds to the Ca^{2+} · target protein complex or to CSA in a mutually exclusive manner, and the Ca^{2+} · target-protein · Cyp complex is required for pore opening. CSA blocks the pore by prevention of association of CyP-D with the target protein. The finding that CyP-18 was not involved according to the photolabelling criteria agrees with our finding that this enzyme is not situated in the matrix compartment. ADP may prevent formation of Ca^{2+} · target-protein · Cyp complex by binding to the free target protein or to CyP.

If this PPIase interacts with the pore protein itself, then it may facilitate a step in pore activation that involves protein-conformational change. In view of electrophysiological data, which shows the rapidity with which the activated pore flickers between open and closed states [37] it seems improbable that it catalyses pore flicker. Nevertheless, it may catalyse formation of the activated state that is capable of flicker. However, any such isomerase-catalysed switch can only be part of the mechanism that involves CyP-D. CSA not only blocks but also reverses pore activation, which suggests that CyP-D (when uncomplexed to CSA) displaces an equilibrium towards an activated state, presumably by binding to the activated state [21]. This is also the model that emerges from the photolabelling data. Since Ca^{2+} is able to depress the binding of PA-CS to CyP-D at 1 μM PA-CS, which is greater than 100-fold of the K_i value for CSA, CyP-D would be expected to associate very strongly with the Ca^{2+} · target-protein complex in the absence of competing CSA.

More speculatively, one could envisage that the isomerase catalyses or stabilizes assembly of a complex pore structure. It has been proposed that closely apposed mitochondria in cells may form tight intermitochondrial junctions [39]. These junctions may involve outer-membrane porins inserted into inner-membrane channels, which allow the conjugated mitochondria to operate as a bioenergetic continuum [39, 40]. It is conceivable that the open pore detected in isolated mitochondria under pseudo-pathological conditions might represent the opening of such a structure in single unconjugated mitochondria. Identification of the CyP-D target and examination of its capacity to interact with porin would enable this notion to be assessed further. There are indications that the inner-membrane adenine-nucleotide translocase is able to form a complex with porin [41] and that it may deform into an open channel under Ca^{2+} stress [35], but there is no evidence yet that the adenine-nucleotide translocase is a target for cyclophilin.

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